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## Technical Report

### a. Scientific and Technical Objectives

Use of resin-based extraction in a closed loop configuration with fermentor-controlled cultivation of phloroglucinol-synthesizing microbes presents numerous complications. Accordingly, microbial synthesis of phloroglucinol has been examined in the absence of resin-based extraction at both 2 L and 20 L scale. Both the relatively short catalytic lifetime of *phlD*-encoded phloroglucinol synthase and the toxicity of phloroglucinol towards *E. coli* are additional challenges encountered during fermentor-controlled synthesis of phloroglucinol. Strategies for improving PhlD specific activity and extending the catalytic lifetime of PhlD in *Escherichia coli* were therefore examined. Activated sludge was explored as a source of microbes that are resistant to the toxicity of phloroglucinol.

### b. Approach

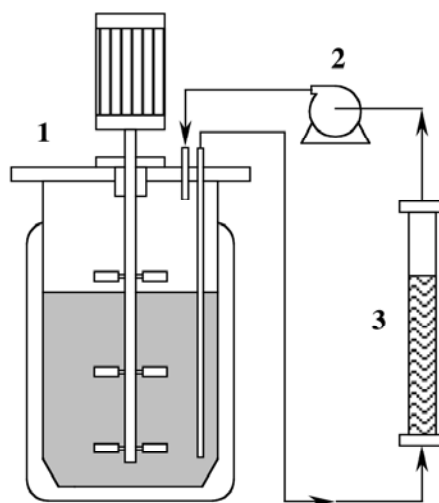
Because *phlD* was isolated from GC-rich *Pseudomonas fluorescence*, codon optimization of the *phlD* sequence for expression in *E. coli* was examined and the impact on the specific activity of PhlD examined in cells cultured under fermentor-controlled conditions. GST, MBP, NusA and SET fusions were constructed with PhlD and examined for catalytic activity and catalytic lifetime. To identify microbes that were resistant to the toxicity of phloroglucinol, activated sludge from the Lansing, MI municipal sewage treatment facility was screened for growth in defined culture medium containing phloroglucinol.

### c. Concise Accomplishments

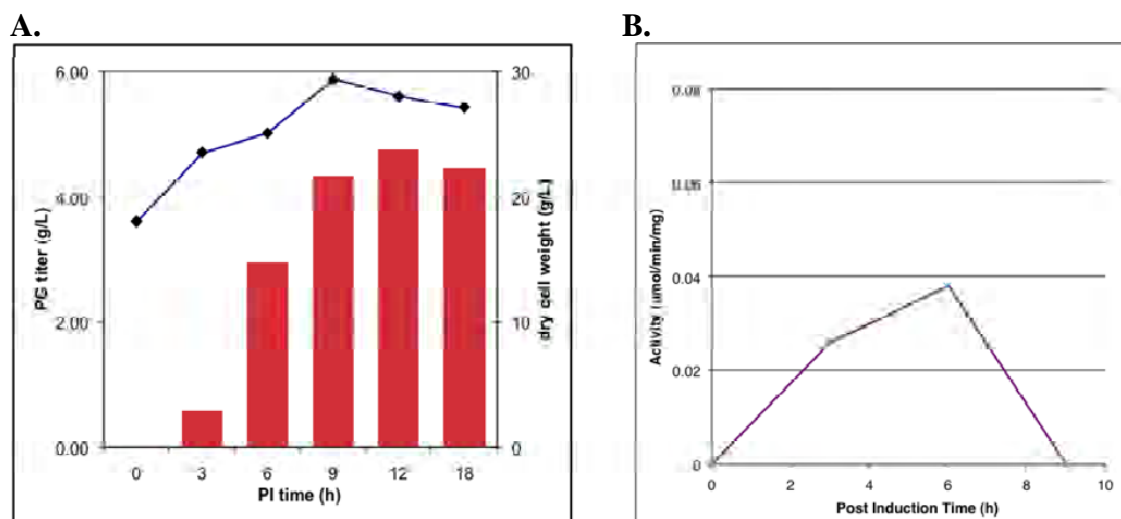
In the absence of resin-based extraction, *E. coli* PG1/pBC2.274 synthesized 4.7 g/L of phloroglucinol at 2 L scale and 3 g/L at 20 L scale under fermentor-controlled conditions. Fusion proteins between GST, MBP and NusA with PhlD were catalytically inactive while the fusion protein of SET with PhlD displayed only trace levels of catalytic activity. Two microbes were discovered that could be grown in defined culture medium containing phloroglucinol at a concentration of 20 g/L. The limit of phloroglucinol's solubility in water is approximately 30 g/L.

### d. Expanded Accomplishments

Phloroglucinol is toxic to *E. coli*. To cope with this toxicity, fermentor culture medium is continuously pumped through Dowex 1 resin (Figure 1). Phloroglucinol in the culture medium binds to the Dowex 1. By using resin-based extraction in a closed external loop with the fermentor (Figure 1), phloroglucinol concentrations in the culture medium can be kept below levels that are toxic to *E. coli*.



**Figure 1. Fermentor-Controlled cultivation with Resin-Based Extraction.** 1. fermentor; 2. peristaltic pump; 3. resin column.

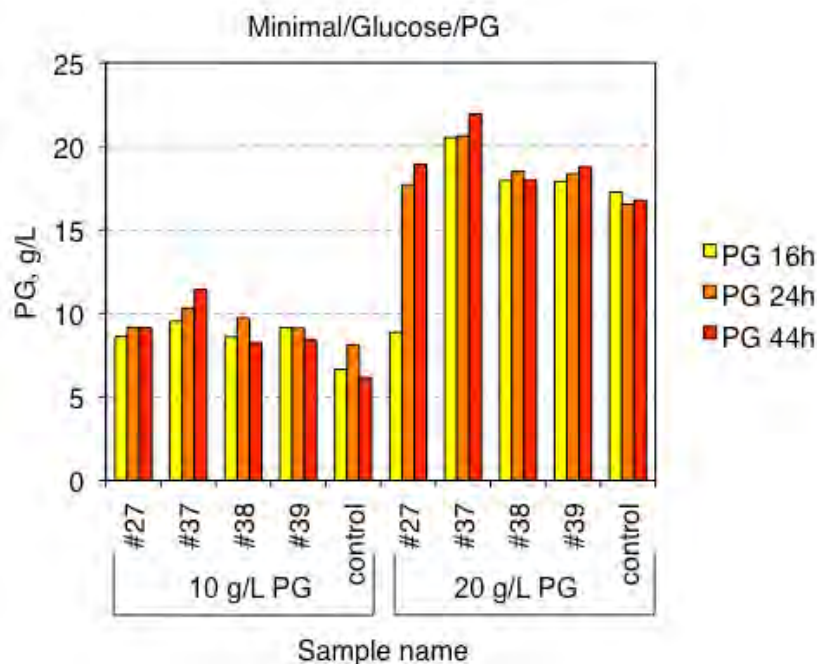


**Figure 1. Cultivation of *E. coli* PG1/pBC2.274 in the absence of Resin-Based Extraction.** A. Dry cell weight (diamonds) and phloroglucinol concentrations (red bars). B. PhlD specific activity.

One challenge with resin-based extraction is that the external loop is a potential source of contamination during large-scale microbial synthesis. Even though Dowex 1 is a resin sold in large volumes, it can become a critical expense item during large-scale microbial synthesis if it can not be reused multiple times after fermentor runs. For these reasons, the impact of running microbial syntheses in the absence of resin-based extraction was examined and proceeded in parallel with optimization of the codon usage in *P. fluorescens phlD* for expression in *E. coli*. The maximum specific activity of wild-type *phlD* cultured under fermentor-controlled conditions in the absence of resin-based extraction was 0.02  $\mu\text{mol}/\text{min}/\text{mg}$ . After optimization of the codons in *phlD* for expression in *E. coli*, the maximum specific activity under fermentor-controlled conditions in the absence of resin-based extraction increased to 0.04  $\mu\text{mol}/\text{min}/\text{mg}$ .

Expression of the *phlD* gene was induced with IPTG addition in the late logarithmic phase of growth. Peak productivity of phloroglucinol is typically observed 3-6 h after induction (Figure 2 A) where the rate of phloroglucinol synthesis was observed to be approximately 1 g/L/h. Phloroglucinol concentrations usually did not increase significantly beyond 9 h after induction of *phlD* expression (Figure 2A). PhlD specific activities were determined on cells withdrawn at timed intervals from the fermentor culture medium (Figure 2B). The highest PhlD specific activity was 0.04  $\mu\text{mol}/\text{min}/\text{mg}$  at 6 h after IPTG induction (Figure 2B). These experiments implicate a correlation between the loss of PhlD activity and cessation of phloroglucinol synthesis. A maximum of 4.7 g/L of phloroglucinol was synthesized at 2 L fermentor scale. Scaling up to 20 L fermentor scale in the absence of resin-based extraction resulted in the formation of 3 g/L of phloroglucinol.

Preliminary evidence has been obtained that PhlD forms inclusion bodies during heterologous expression in *E. coli*. Such in vivo precipitation can have a deleterious impact on both specific activity and catalytic lifetime of PhlD. To deal with this problem, various strategies were examined to form fusion proteins with PhlD in attempt to solubilize and/or stabilize the enzyme. N-Terminal fusions of PhlD were examined with glutathione *S*-transferase (GST), maltose binding protein (MBP) and solubility inducer protein (NusA). No PhlD activity could be detected upon assay of PhlD fusions with GST, MBP, and NusA. C-Terminal fusions were examined with three different solubility enhancement tags (SET1, SET2, and SET3). As opposed to GST, MBP, and NusA, SET is a short peptide chain consisting of 50 amino acids. Only trace levels of activity could be detected for PhlD fusion with SET1 (0.003  $\mu\text{mol}/\text{min}/\text{mg}$ ), SET2 (0.008  $\mu\text{mol}/\text{min}/\text{mg}$ ) and SET3 (0.004  $\mu\text{mol}/\text{min}/\text{mg}$ ).



**Figure 3. Activated Sludge Microbes Resistant to Phloroglucinol Toxicity.**

Activated sludge from the Lansing, MI municipal sewage treatment facility was used as the source of microbes potentially resistant to phloroglucinol. These activated sludge microbes were exposed to steadily increasing concentrations of phloroglucinol in liquid suspension cultures and

on solid medium. This medium was a minimal salts medium containing glucose as the sole source of carbon. The range of phloroglucinol concentrations examined was 0.1 g/L, 0.5 g/L, 2.5 g/L, 10 g/L and 20 g/L. Four well-defined colonies grew in the presence of at least 10 g/L of phloroglucinol and were sent to Accugenix for typing. These four colonies were identified by Accugenix with a high degree of certainty. To make certain that the apparent resistance to phloroglucinol toxicity was not due to degradation of phloroglucinol in the culture medium, the four strains were cultured in the presence of 10 g/L and 20 g/L of phloroglucinol (Figure 3). In the minimal salts medium with glucose as the carbon source, no biodegradation of phloroglucinol was observed.

e. Work Plan

Efforts will continue to manipulate *phlD* at the genetic level to increase its specific activity and its catalytic lifetime under fermentor-controlled conditions. Prospecting will continue for new sources of phloroglucinol synthase activity. The search will also be continued to identify microbes that are resistant to phloroglucinol toxicity. Heterologous expression of *phlD* and attendant synthesis of phloroglucinol will be examined in the recently isolated microbes, which are resistant to 20 g/L of phloroglucinol.

f. Major Problems/Issues

None.

g. Technology Transfer

h. Foreign Collaborations and Supported Foreign Nationals